Identification of potential activators of proteinase-activated receptor-2

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Received 24 September 1997; revised version received 10 October 1997

Abstract In order to identify physiological activators of proteinase-activated receptor-2 (PAR-2), a peptide chloromethane inhibitor (biotinyl-Ser-Lys-Gly-Arg-CH_2Cl) based on the cleavage site for activation of PAR-2 was synthesised and tested with 12 trypsin-like serine proteinases. The second-order rate constant (k_i/K_i) for the formation of the covalent proteinase-inhibitor complex varied by 2×10^5-fold between the proteinases. Biotinyl-Ser-Lys-Gly-Arg-CH_2Cl reacted very rapidly with trypsin, acrosin from sperm and tryptase from mast cells: the k_i/K_i values with these proteinases were greater than 10^5 M^{-1}. s^{-1}. Thus, the specificity of these proteinases matched the sequence of the activation site of PAR-2 and it can be concluded that these proteinases are potential physiological activators of PAR-2.

Key words: Proteinase-activated receptor-2; Peptidyl chloromethane; Tryptase; Acrosin; Affinity label

1. Introduction

Proteinase-activated receptor-2 (PAR-2) [1] is a member of a growing family of G protein-coupled receptors activated by proteolytic cleavage of their extracellular amino-terminal domain [1–3]. Trypsin can cleave the extracellular domain of PAR-2 to generate a tethered ligand analogous to the thrombin receptor [1]. However, the widespread distribution of PAR-2 in tissues not normally exposed to pancreatic trypsin indicates that other physiological activators must exist [4–7].

The activator must be a trypsin-like enzyme capable of cleaving at the Arg^{36}-Ser^{37} bond within the PAR-2 extracellular domain (Ser^{33}-Lys-Gly-Arg-Ser-Leu-Ile-Gly^{40}). Thus, using the nomenclature of Schechter and Berger [8], the physiological activator of PAR-2 should have corresponding subsites (S4-S3-S2-S1) for binding the Ser^{33}-Lys-Gly-Arg^{36} (P4-P3-P2-P1) peptide sequence.

In the present study, we have developed an affinity label based on this sequence for serine proteinases capable of activating PAR-2. A peptidyl chloromethane inhibitor incorporating the PAR-2 cleavage recognition sequence (biotinyl-Ser-Lys-Gly-Arg-CH_2Cl) was tested with a variety of trypsin-like serine proteinases in vitro. The results highlighted three candidate enzymes with favourable kinetic profiles that could cleave the receptor in vivo, namely pancreatic trypsin, mast cell tryptase and acrosin.

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Abbreviations: D-CHT, D-cyclohexyltyrosyl; Bz, benzoyl; Pip, pipecolyl; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

This paper is dedicated to the memory of Professor Stuart R. Stone who died suddenly on 16 December 1996.

2. Materials and methods

2.1. Materials

Human α-thrombin and bovine factor Xa were prepared as described [9,10]. Granzyme A was a gift from Prof. J. Tschopp (Institute of Biochemistry, University of Lausanne, Switzerland). Human plasmin, bovine pancreatic TPCK-treated trypsin, human tissue plasminogen activator (tPA), human plasma kallikrein and TPCK-treated trypsin immobilised on agarose were from Sigma (Poole, UK). Human activated protein C and boar acrosin were produced as described [11,12]. Mast cell tryptase purified from human lung was purchased from Bioass (Diessen, Germany). The activated complement enzyme C1s ($C\overline{1s}$) was purchased from Enzyme Research Laboratories (Swansea, UK). The proenzyme C1r was obtained from Calbiochem (Nottingham, UK) and activated with immobilised trypsin [13]. The chromogenic substrates Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S-2222), D-Ile-Pro-Arg-*p*-nitroanilide (S-2288), D-Phe-Pip-Arg-*p*-nitroanilide (S-2238), D-Val-Leu-Arg-*p*-nitroanilide (S-2266) and D-Pro-Phe-Arg-*p*-nitroanilide (S-2302) were from Chromogenix (Quadratech, Surrey, UK). Methylsulphonyl-D-CHT-Gly-Arg-*p*-nitroanilide (Spectrozyme tPA) was from American Diagnostica Inc. (Greenwich, CT, USA). The peptidyl chloromethane inhibitor biotinyl-Ser-Lys-Gly-Arg-CH_2Cl was synthesised as described [14]. Porcine mucosal heparin was from Grampian Enzymes (Aberdeen, UK). Bovine serum albumin (fraction V) was from Boehringer Mannheim (Mannheim, Germany). Other chemicals were of the highest grade available commercially.

2.2. Kinetic parameters for the inactivation of proteinases by biotinyl-Ser-Lys-Gly-Arg-CH_2Cl

All proteinase assays were performed at 37°C in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.2% (w/v) poly(ethylene glycol) M_r 6000 using a Hewlett-Packard 8452A diode array spectrophotometer [15]. Assays for activated protein C also included 5 mM $CaCl_2$ and 0.1% (w/v) bovine serum albumin and tryptase assays contained 10 μg/ml heparin. Estimates for the inactivation rate constant for biotinyl-Ser-Lys-Gly-Arg-CH_2Cl were determined by using progress curve kinetics [16]. Each progress curve experiment consisted of 5–7 assays with different concentrations of biotinyl-Ser-Lys-Gly-Arg-CH_2Cl and an appropriate peptidyl-*p*-nitroanilide substrate (400 μM) for the proteinase under study. The peptidyl chloromethane inhibitor was added immediately before the assay was started, and the reaction was initiated by addition of the proteinase at a concentration such that the velocity in the absence of the inhibitor (v_o) was in the range of 0.5–1.0 μM min^{-1}. An absorption coefficient of 9920 M^{-1} cm^{-1} at 405 nm for *p*-nitroaniline was used in the calculation of the amount of product formed [17]. The concentrations of peptidyl-*p*-nitroanilide substrates were determined spectrophotometrically at 342 nm using an absorption coefficient of 8270 M^{-1} cm^{-1} [17]. The Michaelis-Menten equation was fitted by non-linear regression to the initial rates of hydrolysis of peptidyl-*p*-nitroanilide substrates, over a 10-fold range of substrate concentrations to yield estimates for the K_m values (Table 1).

The inactivation of a serine proteinase (E) by a peptidyl chloromethane inhibitor (I) which competes with substrate (S) in the active site of the enzyme can be described by the following scheme, where K_i is the dissociation constant for the initial non-covalent complex (EI*), and k_i is the rate constant for the conversion of the initial complex

PII S0014-5793(97)01298-2

into the covalent complex (EI) in which the active-site histidine is alkylated.

$$E + I \rightleftharpoons^{K_i} EI* \xrightarrow{k_i} EI$$

For this scheme, the progress curve for the production of *p*-nitroaniline (P) due to substrate cleavage will be given by:

$$[P] = v_s t + \frac{v_z - v_s}{k'}(1 - e^{-k't}) \quad (1)$$

where v_z is the initial velocity at $t = 0$, v_s is the final steady-state velocity and k' is the observed first-order rate constant for the formation of the inhibited proteinase. When all E has been inactivated [P] will be constant with time (i.e. $v_s = 0$) and Eq. 1 simplifies to:

$$[P] = \frac{v_z}{k'}(1 - e^{-k't}) \quad (2)$$

For this two-step mechanism of irreversible inhibition, the rate constant k' is related to k_i by the following relationship:

$$k' = \frac{k_i[I]}{[I] + K_i(1 + [S]/K_m)} \quad (3)$$

The value of k' will be dependent on [I] and [S]. In all our experiments we observed a linear dependence of k' on [I], implying that $[I] \ll K_i(1 + [S]/K_m)$. This will occur when a significant concentration of the initial complex does not form. In this case, the dependence of k' on [I] is given by:

$$k' = \frac{(k_i/K_i)[I]}{1 + [S]/K_m} \quad (4)$$

where k_i/K_i is the second-order rate constant for the formation of the inactivated complex (EI) and is equivalent to the specificity constant (k_{cat}/K_m) for substrate hydrolysis. The relationship given in Eq. 4 was substituted into Eq. 2 and the resulting expression was fitted by non-linear regression to a progress curve consisting of 5–7 curves with different concentrations of biotinyl-Ser-Lys-Gly-Arg-CH_2Cl to yield estimates for k_i/K_i.

3. Results and discussion

In order to identify possible activators of PAR-2, a peptidyl chloromethane based on the activation site of PAR-2 was synthesised and tested with a number of serine proteinases. The activation site of human PAR-2 consists of the following sequence: Ser^{33}-Lys-Gly-Arg-Ser-Leu-Ile-Gly^{40}, where cleavage at the Arg^{36}-Ser^{37} bond leads to receptor activation [4]. Thus the affinity label biotinyl-Ser-Lys-Gly-Arg-CH_2Cl should allow the identification of potential activators. After binding of the peptide to the active site, the chloromethane moiety alkylates the active-site histidine resulting in covalent linking of the inhibitor to the proteinase [18]. The biotin was incorporated to allow subsequent detection and purification of labelled proteinases from tissue extracts [14].

The second-order rate constant k_i/K_i for the formation of the covalent proteinase-inhibitor complex is equivalent to the specificity constant k_{cat}/K_m for substrate reactions and thus measures how closely the sequence of the inhibitor matches the specificity of a particular proteinase. Since the sequence of the peptidyl chloromethane corresponds to that of the PAR-2 activation site, the k_i/K_i value also measures whether the specificity of the proteinase is consistent with a potential role as an activator of the receptor.

Biotinyl-Ser-Lys-Gly-Arg-CH_2Cl displayed k_i/K_i values ranging from 3.1×10^5 M^{-1} s^{-1} with acrosin to 1.8×10^1 M^{-1} s^{-1} with granzyme A (Table 1). The inhibitor reacted most rapidly with acrosin, tryptase and trypsin ($k_i/K_i > 10^5$ M^{-1} s^{-1}). Factor Xa was the only other proteinase tested that exhibited a relatively high k_i/K_i value with biotinyl-Ser-Lys-Gly-Arg-CH_2Cl (2×10^4 M^{-1} s^{-1}). A number of proteinases (thrombin, activated complement factor C1r ($\overline{C1r}$), plasmin, tissue plasminogen activator (tPA) and plasma kallikrein) reacted relatively slowly with biotinyl-Ser-Lys-Gly-Arg-CH_2Cl ($k_i/K_i = 10^2$–10^3 M^{-1} s^{-1}). At the bottom end of the reactivity scale were activated complement factor C1s ($\overline{C1s}$), activated protein C and granzyme A which had k_i/K_i values less than 50 M^{-1} s^{-1} with biotinyl-Ser-Lys-Gly-Arg-CH_2Cl.

The observed rapid inhibition of pancreatic trypsin with biotinyl-Ser-Lys-Gly-Arg-CH_2Cl ($k_i/K_i = 1.12 \times 10^5$ M^{-1} s^{-1}) correlates with the ability of trypsin to activate PAR-2 in vivo [1]. PAR-2 is highly expressed in the pancreas and gastrointestinal tract, where it may be activated by pancreatic trypsin [4,5]. In support of this hypothesis, trypsin activation of PAR-2 stimulates the contraction of gastric smooth muscle and the secretion of amylase from pancreatic acini [5,19]. However, the expression of PAR-2 is not restricted to the gastrointestinal tract and, thus, other activators must exist. The present study has identified two other potential activators that reacted faster with biotinyl-Ser-Lys-Gly-Arg-CH_2Cl than trypsin, namely mast cell tryptase and acrosin from sperm.

Tryptase is a tetrameric trypsin-like enzyme present in mast cells, which is released in an active state upon mast cell degranulation [20]. The active tetrameric form is stabilised by heparin and in the absence of heparin, the tryptase subunits dissociate to inactive monomers [21]. The observed rapid in-

Table 1
Second-order rate constant (k_i/K_i) for the reaction of biotinyl-Ser-Lys-Gly-Arg-CH_2Cl with different serine proteinases

	k_i/K_i (M^{-1} s^{-1})	Substrate	K_m (μM)
Acrosin	$3.09 \pm 0.01 \times 10^5$	S-2288	12
Tryptase	$1.48 \pm 0.02 \times 10^5$	S-2288	188
Trypsin	$1.11 \pm 0.01 \times 10^5$	S-2288	28
Factor Xa	$2.00 \pm 0.02 \times 10^4$	S-2222	47
Thrombin	$1.84 \pm 0.02 \times 10^3$	S-2238	4
Activated C1r ($\overline{C1r}$)	$6.33 \pm 0.18 \times 10^2$	S-2288	114
Plasmin	$6.08 \pm 0.12 \times 10^2$	S-2288	1110
Tissue plasminogen activator	$4.34 \pm 0.01 \times 10^2$	Spectrozyme tPA	345
Plasma kallikrein	$3.99 \pm 0.04 \times 10^2$	S-2302	272
Activated C1s ($\overline{C1s}$)	$4.22 \pm 0.08 \times 10^1$	S-2288	1800
Activated protein C	$2.15 \pm 0.01 \times 10^1$	S-2266	222
Granzyme A	$1.77 \pm 0.04 \times 10^1$	S-2302	1100

Assays were performed and data analysed as described in Section 2. The estimates of k_i/K_i obtained are given with the standard errors derived by non-linear regression analysis. The substrates used in the assays and their K_m values are also shown.

activation of tryptase by biotinyl-Ser-Lys-Gly-Arg-CH_2Cl ($k_i/K_i = 1.5\times10^5$ M^{-1} s^{-1}) is consistent with previous studies of its specificity using synthetic substrates: Lys-Gly-Arg-*p*-nitroanilide was among the best of the substrates tested [22]. The rapid inhibition of mast cell tryptase by biotinyl-Ser-Lys-Gly-Arg-CH_2Cl in vitro is in agreement with the observation that tryptase is another physiological activator of PAR-2 in vivo [23]. Tryptase is able to cause an increase in cytosolic Ca^{2+} when added to human umbilical vein endothelial cells or keratinocytes which both express PAR-2 [23], eliciting a similar response when PAR-2 is activated by either trypsin or an agonist peptide in these two cell types [6,7].

Acrosin is a trypsin-like serine proteinase that is found in large quantities within the acrosomal vesicle of mammalian spermatozoa [24]. Like trypsin, acrosin cleaves after arginine or lysine residues and has a preference for arginine over lysine in the P1 position [25]. Acrosin was also rapidly inactivated by biotinyl-Ser-Lys-Gly-Arg-CH_2Cl with a k_i/K_i value of 3.1×10^5 M^{-1} s^{-1} (Table 1). Acrosin was inactivated faster than trypsin or mast cell tryptase, which have both demonstrated to be physiological activators of PAR-2. This result implies that under appropriate conditions acrosin is a potential activator of PAR-2. In this respect, it is interesting to note that Northern blot analysis demonstrated PAR-2 expression in both the male and female reproductive tract: PAR-2 was expressed in prostate and ovary but not in the testis [4,5].

In conclusion, the results of the present study have identified acrosin, tryptase and trypsin as potential physiological activators of PAR-2. Biotinyl-Ser-Lys-Gly-Arg-CH_2Cl reacted very rapidly with trypsin, tryptase and acrosin compared with the other proteinases tested. The high degree of selectivity exhibited by biotinyl-Ser-Lys-Gly-Arg-CH_2Cl for both acrosin and tryptase provides a useful affinity label for the study of these two trypsin-like serine proteinases. It should also be possible to use the biotinylated affinity label in future studies in conjunction with SDS-PAGE and Western blotting techniques, for the detection and partial characterisation of other potential activators of PAR-2.

Acknowledgements: We thank the British Heart Foundation for financial support, and Dr. C. Graham Knight for his careful reading of the manuscript.

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